Allergens

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The problem of isolation and characterization of allergens is complicated for many reasons, some of which are discussed. Included are: the present state of classification of allergens; the significance of the original elucidation of the polysaccharidic protein nature of the cottonseed and castor bean allergens in relation to isolation and chemistry of allergens in general; and the related concept that chemically different compounds may share common antigenic and allergenic determinants. Food allergy is especially complicated in that the products of digestion may trigger an allergic response. Based on demonstration of 12 new antigens generated by pepsin hydrolysis of milk proteins, we have suggested that the body immune system may be exposed to at least 100 new antigens, all of which are potential allergens, on ingestion of milk. Our results may explain why foods, in many cases, do not give a skin reaction on persons who give an immediate-type allergic response on ingestion of the food.

The objectives of this review are to discuss: problems involved in studying the chemistry of allergens; emerging concepts of the chemical nature of allergens and their determinants; clarification of the significance of work on oilseed allergens; and current research at Dairy Products Laboratory (DPL) on attempted elucidation of the mechanism of the allergic response to ingested milk proteins in particular and other foods in general.

The term "allergie" was used first by von Pirquet (1906) to denote an altered capacity of a human to react to a second injection of horse serum. Since that time "allergy" has generally been used to describe all forms of hypersensitivity in man. An allergen may be defined as "an ordinarily harmless substance present in the diet or environment, capable of producing such diseases as asthma, hay fever, eczema, and gastrointestinal upsets upon contact with a previously sensitized person." Allergy is the body response to an allergen antibody reaction which triggers the release of chemical mediators of hypersensitivity, namely histamine, serotonin, and acetylcholine, as well as larger compounds, SRS (slow reacting substance), and the plasma kinins (Austen, 1965). There are essentially three types of allergy. In atopic allergy, immediate-type symptoms appear in a few minutes up to 60 min after exposure. This type of reaction is initiated by the specific reaction of allergen with reagin [also called skin-sensitizing antibody, homocytotropic antibody or IgE (Ishizaka and Ishizaka, 1970)]. In delayed allergy, symptoms appear in from a few up to 96 hr after exposure. This reaction is initiated by specific reaction between allergen and small lymphocytes (Raffel, 1965). Anaphylactic-type allergy may occur in seconds to minutes after exposure with violent, sometimes fatal, symptoms. This reaction is initiated by specific reaction between allergen and IgG or IgE antibodies. This paper is mainly concerned with immediate-type allergy.

Reported overall incidence of allergy varies, but 10% of the general population is cited most often.

A comprehensive review of the chemistry of allergens is beyond the scope of this paper both because of space limitations and because recent publication of a monumental, critical review containing 558 references would make such an attempt redundant from a literature reference standpoint. Berrens (1971), in a monograph of 298 pages titled "The Chemistry of Atopic Allergens," has reviewed the subject of allergens in eight categories, namely the pollens, including timothy and cocksfoot (Augustin and Hayward, 1962), ryegrass (Johnson and Marsh, 1966a), alder (Herbertson *et al.*, 1958), ragweed, the greatest single cause of allergy from a natural source in the U. S. (Goldfarb, 1968; King *et al.*, 1967; Richter and Sehon, 1960; Robbins *et al.*, 1966; Underdown and Goodfriend, 1969); allergens from vegetable dusts and fibers, including ipecac (Berrens and Young, 1963), liquorice (Berrens, 1964), pyrethrum (Zucker, 1965), kapok (Berrens, 1966a; Coulson *et al.*, 1944), and cotton linters (Berrens and Versie, 1967; Coulson and Stevens, 1940); allergens from seeds, mainly cottonseed (Spies *et al.*, 1940b, 1960) and castor beans (Panzani and Layton, 1963; Spies, 1967; Spies and Bernton, 1962; Spies and Coulson, 1964); allergens from epithelial tissues such as horse dandruff (Stanworth, 1957), human dandruff (Berrens *et al.*, 1966), feathers (Berrens,

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1968a), and insects, exclusive of biting and stinging varieties (Shulman, 1968); molds (Barker *et al.*, 1967; Prince *et al.*, 1961); the ubiquitous house dust allergens (Berrens, 1970); allergens from foods (Bleumink, 1970), including tomato (Bleumink *et al.*, 1967), cow's milk (Bleumink and Young, 1968; Goldstein and Heiner, 1970; Hanson and Johansson, 1970; Spies, 1973), egg (Bleumink and Young, 1969), and fish (Aas and Elsayed, 1969); and finally miscellaneous allergens, including *Ascaris* (Strejan and Campbell, 1968) and other parasites, various seeds and nuts (Spies *et al.*, 1951), vegetable gums (Gelfand, 1943), low-molecular weight allergens such as penicillin (Levine and Redmond, 1969), and chocolate and cocoa. The selected references are for the convenience of the reader and are not intended as an evaluation of their relative importance in the field. Berrens (1971) has also attempted the physicochemical classification of allergens, discussed the functional characteristics of allergens and his "... proposedly universal structural determinant of lysine–sugar conjugation among atopic allergens."

SOME SPECIAL PROBLEMS IN THE ISOLATION AND CHEMICAL CHARACTERIZATION OF ALLERGENS

The problems of isolation and chemical characterization of allergens are complicated by many factors. Following are some of the more important of these problems. Although animal experimentation is helpful, ultimate evaluation of allergens requires human testing procedures, which are inaccurate, difficult to interpret, and influenced by variations in patient sensitivity. Multiple symptoms may be produced by exposure to some allergens, *e.g.*, allergic reaction to a milk protein may be one or more of vomiting, diarrhea, abdominal pain or colic, rhinitis, asthma, dermatitis, urticaria, anaphylaxis, and central nervous and other symptoms. Biological tests for allergens are more sensitive than chemical tests for constituents; hence rigid isolation and characterization procedures are required to ensure that the allergenic activity measured is inherent and not owing to difficultly removable trace contaminants. Some patients are sensitive to different components of a natural source, while others are sensitive to more than one component of a natural source. Hence, measured allergenic activity of less than 100% pure preparations may be the sum of activities of two or more allergens. In food allergy the skin reaction is particularly unreliable for evaluation of isolated fractions because some persons who give an immediate-type clinical response to ingestion of a food may not give a positive skin reaction and vice versa. Uncertainty regarding the relationship between antigenic and allergenic determinants makes questionable the correlation of serological and other manifestations of antigenicity with allergenicity. However, the distinction between allergen and antigen may lie more in a different human response to an antigenic determinant than in structural or chemical differences in antigenic and allergenic determinants. Not the least of these complications is that chemically distinct compounds sometimes contain the same allergenic and/or antigenic determinants, as shown by their specific reactions with reagins and by specific antibody–antigen reactions. Chemically distinct compounds with shared determinants may occur in protein mixtures or in protein(s) containing varying amounts of polysaccharidic carbohydrate or possibly in other unknown compositional or configurational variations.

Most of the work on the chemistry of allergens has been done on their isolation and characterization. Considering the foregoing complexities, it is not surprising that only a few attempts have been made to classify allergens in general on the basis of their physicochemical properties. Augustin (Augustin, 1959; Augustin and Hayward, 1962) suggested that allergens as a class distinguished themselves from antigens by some special though unknown structural features. Stanworth (1963) noted the narrow range of molecular weights of several allergens (25–40,000). Berrens (1962) proposed that allergenic properties may originate by Maillard-type reactions between proteins and reducing sugars, thereby forming structural sites of lysine–sugar complexes. Berrens has devoted extensive research to establishment of this hypothesis and has assembled impressive though inconclusive evidence concerning it. One of the principal objections to this hypothesis is that it has not been reconciled satisfactorily with the property of immunological specificity, which is a cornerstone of antigen–antibody and allergen–antibody reactions.

Berrens (1971) has laudably attempted classification of the sufficiently characterized allergens on the basis of their compositional and physicochemical properties which he has tabulated and discussed in his monograph. The most important generalizations are discussed below.

Glycoprotein Nature of Allergens. The hexose + pentose contents of 24 allergens tabulated by Berrens (1971) ranged from 0 to 77% and their nitrogen contents ranged from 0.57 to 20.4%. Based on these data, Berrens (1971) summarized: "For simplicity it is safe to state that atopic allergens fall into a category of glycoproteins of widely varying overall composition."

Berrens has discussed the work of the U. S. Department of Agriculture group, consisting of Bernton, Coulson, Spies and Stevens, on isolation and chemical, immunological, and clinical characterization of cottonseed, castor bean, and other oilseed allergens. This discussion, although remarkably detailed, unfortunately contains some factual errors as well as questionable interpretations; see the Supplementary Material Available paragraph at the end of this paper. Because of the relationship of our work to the origination and development of the concept of the polysaccharidic protein nature of the principal allergen(s) of cottonseed, castor beans, and other oilseeds, together with the related idea that chemically different compounds share antigenic and/or allergenic determinants, it seems appropriate to summarize briefly that part of the work at USDA that has a bearing on this subject. The general relationship of this concept to allergen isolation studies will be evident in the discussion which follows.

The object of our first study was isolation and characterization of the allergen(s) of cottonseed meal. General opinion at that time was that a specific allergenic activity resided in a single chemical entity, probably protein in nature. Fractions CS-1 (Spies *et al.*, 1939) and CS-1A (Spies *et al.*, 1940b) were isolated from cottonseed meal. CS-1A contained the principal allergen(s) of cottonseed immunologically distinct from other allergens (Bernton *et al.*, 1942; Spies *et al.*, 1942) and from other antigens (Coulson, *et al.*, 1941, 1943a) in the seed. Components of CS-1A appeared to be preformed native proteins (Coulson *et al.*, 1941, 1943a,b). The CS-1A isolation procedure was generalized to some extent by its use in isolation of the principal allergen(s) of castor beans, CB-1A (Spies and Coulson, 1943; Coulson *et al.*, 1950), and similar "1A" fractions from several other oilseeds and nuts (Spies *et al.*, 1951), all of which were polysaccharidic proteins.

It was recognized early that CS-1A was a complex mixture (Spies *et al.*, 1940a). Six polysaccharidic protein fractions containing from 0.9 to 35% of chemically combined carbohydrate were isolated in a high-voltage electrophoretic fractionation of 400 g of CS-1A. On the basis of this study we wrote (Spies *et al.*, 1941): "The evidence presented indicates that CS-1A is a mixture containing specifically active protein and active compounds of this protein chemically combined with varying amounts of polysaccharide." At this point our thinking changed, and our efforts became directed toward demonstration that chemically different components of CS-1A contained the same antigenic and allergenic determinant groups.

An active carbohydrate-free allergen, CS-60C, was isolated from CS-1A (Spies and Umberger, 1942). CS-60C was not homogeneous and we stated: "It seemed more probable that CS-60C represented a mixture of proteins whose structural variations were too slight to permit effective chemical fractionation or perhaps too slight to impart immunological identity even if they could be separated." Prolonged dialysis of one of the electrophoretic fractions, CS-56R (35% carbohydrate) yielded a fraction, CS-56RExD, which contained 87% combined carbohydrate (Coulson *et al.*, 1949). We thus had separated from CS-1A a series of compounds containing from 0 to 87% polysaccharide. Using these compounds, including CS-1A and others, it was demonstrated that the combined polysaccharide enhanced the guinea pig anaphylactic sensitizing capacity of the protein but did not determine the antigenic specificity nor influence the anaphylactic shocking capacity of the protein (Coulson and Spies, 1943a; Coulson *et al.*, 1949). Furthermore, the antigenic specificity of CS-60C was the same as that of CS-56RExD as shown by Schultz–Dale tests. The guinea pig used was immunized with CS-1A. One uterine horn responded maximally to challenge with CS-60C, which desensitized it to further test with CS-56RExD, and likewise the other uterine horn of this guinea pig responded maximally to challenge with CS-56RExD, which desensitized it to test with CS-60C. This specificity relationship was true for the other CS-1A subfractions (Coulson *et al.*, 1949). It was concluded from all of these studies that not only did the combined polysaccharide enhance the sensitizing capacity of the protein but also it increased the molecular weight and influenced electrophoretic mobilities and certain other properties without conferring a distinct specificity or influencing the anaphylactic shocking capacity of the protein.

Thirty years later, after attempting to subfractionate his house dust allergenic fraction E, Berrens (1971) wrote: "Attempts to subfractionate this material have consistently failed to provide a more potent or more specific product. The total outcome of these efforts has been a mere division of fraction E into several glycoprotein subfractions exhibiting virtually identical or slightly decreased allergenic activity." This conclusion is compatible with the net results of our fractionations of CS-1A.

Electrophoretic Mobilities and Isoelectric Points. Berrens (1971) has tabulated the direction of migration of 26 purified allergens and the apparent isoelectric points of 18 of these allergens. He states that the evidence confirms the general contention that atopic allergens are acidic glycoproteins of fast anodic migration velocity in the pH range 7–9. The apparent isoelectric points fall within the range of 2 to 5.5. According to Berrens (1971): "Notable exceptions are the basic proteins from cottonseed, and castor beans described by Spies and his group [Chapter IV] and ragweed Ra. 3 of Underdown and Goodfriend."

Berrens (1971) has explained the acidity of allergens, the gradient of increasing electrophoretic mobilities and decreasing isoelectric points in a series of purified allergens by progressive blockade of ϵ-amino groups of lysine side chains in peptide moieties by aldose sugars, which results in an increase in the contribution to charge by acidic amino acid residues. He also suggests that the Amadori configuration of the lysine–sugar grouping in one of its enolic forms may add to acidity by the negative charge on the hydroxyl group in the sugar residue. He offers this as a plausible explanation for multiple electrophoretic forms of ryegrass pollen (Johnson and Marsh, 1965, 1966b) and for diffuse electrophoretic zones obtained with allergenic preparations from house dust, ipecac, human dandruff, kapok, etc.

Although Berrens (1971) has cited cottonseed and castor bean allergens as exceptions to the foregoing generalizations, actually the polysaccharidic proteins obtained by electrophoresis of CS-1A had isoelectric points from approximate pH values 3 to 9.4, depending on their carbohydrate content. Thus, in the preparative electrophoretic fractionation of CS-1A (Spies *et al.*, 1941), six fractions were obtained from six cells into which they had migrated and remained in electrophoretic equilibrium. These fractions, designated CS-51R, CS-52R, CS-53R, CS-54R, CS-55R, and CS-56R, containing 1, 1, 3, 6, 24, and 35%, respectively, of polysaccharidic carbohydrate, were isolated from cells in which pH values were 9.4, 6.4, 5.7, 5.0, 4.1, and 3.0, respectively (Spies *et al.*, 1941). Our explanation was: "The electrophoretic technique employed effects separation of these substances because the net charge of the protein polysaccharidic components would be different from that of the protein alone. If the polysaccharide contained acidic groups or was combined with basic groups of the protein, then the greater the proportion of combined carbohydrate the lower would be the pH of the isoelectric point of that compound." Berrens (1965) indicated that the coupling of sugar to lysine has more or less been suggested by our observations. Berrens (1971) has commented further on this point. As pointed out above, all of these polysaccharidic proteins had the same antigenic specificity and essentially the components of CS-1A must have had the same allergenic specificity as shown by reagin neutralizing capacities of ion-exchange fractions in which fraction (CS-13E) 5F6 (estimated carbohydrate, 0.5%), representing only 1.25% of CS-1A, neutralized the serum of a cottonseed-sensitive patient to further test with CS-1A (Spies *et al.*, 1960).

The castor bean allergen CB-1A, like CS-1A, contained active protein (Spies *et al.*, 1944a,b) and polysaccharidic protein complexes whose migration was influenced by the carbohydrate yet contained common antigenic determinants. For example, CB-1A fraction E1 (26% carbohydrate) gave a diffuse pattern, part of which migrated toward the anode, whereas CB-1A fraction D5 (1% carbohydrate) migrated toward the cathode on cellulose acetate at pH 8.6. Electrophoretically, E1 and D5 appeared to be chemically distinct. By immunoelectrophoresis in a specially designed experiment which took advantage of their different electrophoretic behavior, it was shown that fractions E1 and D5 contained a common major antigenic determinant (Spies and Coulson, 1964, Figures 10 and 11).

The carbohydrate-free allergenic fraction from CB-1A, CB-65A (Spies *et al.*, 1944a,b), also contained distinctly migrating components possessing a common antigenic specificity (Morris *et al.*, 1965). For other papers bearing on the subject of chemically different components of CB-1A containing common antigenic specificities as well as the total number of minor specificities contained in CB-1A, see Spies and Barron (1966) and Spies (1967).

Molecular Weight of Allergens. Stanworth (1963) tabulated sedimentation coefficients of 2.2S to 3.8S for six purified allergen preparations and commented on the narrow range of indicated molecular weights. Berrens (1971) recorded sedimentation coefficients for 19 purified allergens and the estimated molecular weights of some of them. He observed that the sedimentation constants of most of them fell in the range of 2–4S (estimated molecular weight range 25–40,000). A sedimentation coefficient of 4 was a definite upper limit. The lower limit was more flexible as the value for some pollen and fish allergens was less than 2S, for caddis fly it was 0.72S (Shulman *et al.*, 1963), and for cottonseed allergens it ranged from 1 to 1.6S (Spies *et al.*, 1959). Explanations for this restricted molecular size of allergens are speculative.

Solubilities of Allergens. The low-molecular weight glycoprotein allergens are soluble in water. The salt concentrations required for complete precipitation of polydisperse glycoprotein allergens depend on precipitation of both protein-rich to carbohydrate-rich components. Berrens (1971) states that in going toward the carbohydrate-rich and increasingly disperse region, precipitation re-

quires high salt concentration. For this reason the extended salt concentration of 0.3 to 0.8 saturated ammonium sulfate is indicated for complete precipitation of the glycoprotein allergens. Ethanol was used as precipitant in studying the cottonseed polydisperse polysaccharidic protein allergens. Similar to use of ammonium sulfate, concentrations up to 80% ethanol were required for precipitation of the carbohydrate-rich components, the protein-rich components being precipitated at lower concentrations.

In studying the allergenic activities of other proteins, globulin for example, appropriate salt concentrations are required for solution and precipitation. Glutelins and denatured proteins require dilute alkali for solution where there is danger of destruction of activity.

Stability. Reports on the stability of allergens to heat and pH are conflicting. Richter and Sehon (1960) reviewed the history of reports on the stability of ragweed pollen allergens which ranged from 90% destruction within 1 hr at 56° to no loss after 1 hr at 100°. Berrens (1971) states that there is general agreement on the thermostability of atopic allergens. Allergenic potency as a rule remains unaffected by heating solutions at 100° for periods exceeding 1 hr. He states that similar conditions cause the majority of protein antigens to lose their immunological characteristic and native antigenic structure; but even though the antigenic determinants of allergen molecules may undergo modification or be destroyed during the heat treatment, the allergenic determinants appear to be exceptionally stable.

Among the most stable allergens are CS-1A from cottonseed and CB-1A from castor beans. Heating at 100° for 1 hr was part of the isolation procedure, and this treatment did not destroy nor diminish their native preformed antigenic structures (Coulson *et al.*, 1943a, 1950). CS-1A components even retained cutaneous activity and reactivity in passive transfer tests with serum from a cottonseed-sensitive patient after being refluxed (above 100°) in 0.1 *N* sulfuric acid for 4 hr (Spies *et al.*, 1941). The acid-treated CS-1A components also were antigenic and possessed reagin-neutralizing capacity, although it was of decreased potency (Coulson and Spies, 1943b).

Spies *et al.* (1962) determined the conditions of temperature, time and pH for the inactivation of CB-1AE from castor beans, as measured by the effect on cutaneous activity, reagin-neutralizing capacity, and immune precipitating capacity. Heating for 1 hr at 110° at pH 5.9 had no effect on the immune precipitating capacity. At pH 5.9, it required heating for 1 hr at 150° and 140° to destroy completely the precipitating and reagin-neutralizing capacities, respectively. CB-1AE showed remarkable stability to heating in alkaline solution. Thus, at 100° and 120° at pH 12 or over, the reagin-neutralizing capacity was lost in 32 and 8 min, respectively, and the cutaneous activity in 32 and 8 min, respectively, using samples tested after two-fold time increments, starting at 1 min. There was a difference in stabilities of reagin-neutralizing capacity and precipitating capacity which depended on the pH. At pH values of 5.9 to 8.7 the precipitating property was more stable to heat than reagin-neutralizing property. But, at pH 10.8 to 11.9 the reverse was true. The results of this study showed that determination of loss of precipitating capacity of the principal castor bean allergen(s) cannot be used as a criterion in the determination of *complete* destruction of its allergenic properties. However, loss of precipitating capacity is a convenient indicator of nearly complete destruction of allergenicity.

Digestibility by Enzymes. Enzymes were early used to determine the chemical nature of allergens. Later, when it was established that most allergens were proteins or glycoproteins, susceptibility to enzyme digestion was used to locate active sites in the molecule. Susceptibility of allergens to proteolysis varies. Native antigen E, the principal allergen of ragweed pollen, is not digested by trypsin, chymotrypsin, and papain but is readily digested by pepsin at pH 2 because of rapid acid denaturation at that pH according to King *et al.* (1967). Allergens from ryegrass pollen were readily hydrolyzed by trypsin and chymotrypsin with loss of cutaneous activity, but treatment of ryegrass allergen I-B with the carbohydrases, cellulase, β-glucosidase, β-galactosidase, β-amylase, or lysozyme showed no loss in activity (Johnson and Marsh, 1966a). The cottonseed allergen (CS-13-Endo, 7.7% carbohydrate) was readily hydrolyzed by trypsin with loss of activity, by chymotrypsin with partial loss of activity, and by pepsin and carboxypeptidase with retention of activity (Spies *et al.*, 1953).

Berrens (1968b) studied the action of trypsin, chymotrypsin, and pancreatic kallikrein on several allergens which proved to be resistant to proteolysis. He correlated the resistance of certain glycoprotein allergens to digestion by trypsin and chymotrypsin to the number of lysyl residues blocked as sugar conjugates. He showed that these glycoprotein allergens were competitive inhibitors of the proteolytic activities of trypsin and chymotrypsin but not of kallikrein. The esterolytic activity of the three enzymes was unimpaired by the presence of glycoprotein allergens. Bleumink and Young (1968) similarly correlated a decrease in digestibility of β-lactoglobulin reacted with varying amounts of lactose to an increase in the lysine–lactose groupings in the molecule.

Optical Properties. The ultraviolet absorption spectra of protein and glycoprotein allergens in the 250–300 nm range, in the main, are due to their aromatic amino acid and cystine contents (Greenstein and Winitz, 1961). However, ultraviolet absorption (Berrens and Bleumink, 1965, 1966) and fluorescent (Berrens, 1966b, 1967) optical properties of allergens have been traced to the lysine–sugar conjugate site.

Maillard reactions between proteins and aldoses were shown to create a new chromophore causing a new low-intensity maximum at 305–310 nm in the resulting glycoprotein molecule. The 310-nm maximum was attributed to the 1–2 enolic tautomer of the 1-deoxy-2-ketose residue resulting from the Amadori rearrangement of the lysine–sugar conjugate. Berrens (1971) used the ratio of extinction coefficients E_{305}:E_{280} (1%) for estimation of the mean number of lysine–sugar sites in a number of allergen molecules and attempted an arrangement of purified allergens based on the extent of their lysine substitution.

The other optical property is the blue or blue-green fluorescence in the 430–490 nm region produced by irradiation of glycoprotein allergen solutions at 313–365 nm. A study of the kinetics of the fluorescent emission during the Maillard reaction showed that blue fluorescent compounds are generated in the initial phases of the reaction. Fluorophores can be detected at 430–450 nm at earlier stages of the reaction than can the chromophores at 305 nm. However, fluorescence reaches a maximum and then gradually declines, whereas the ultraviolet absorption at 305 nm continues to rise steadily as a result of secondary degradation or condensation reactions at the expense of the highly reactive Amadori product. According to Berrens, sequential arrangement of allergens on the basis of their fluorescent emission implies a refinement over the arrangement on the basis of 305-nm absorption in that fluorescence apparently is determined by the number of lysine–sugar residues in highly reactive 1–2 enolic configuration.

Berrens and Bleumink (1965) noted that the ultraviolet absorption spectrum of the carbohydrate-free cottonseed allergen CS-60C (Spies and Umberger, 1942) possessed the absorption characteristics of a glycoprotein allergen rather than those of a carbohydrate-free protein. To reconcile CS-60C, a potent allergen, with their hypothesis of the lysine–sugar as the universal allergenic determinant, these authors have speculated that ε-amino groups of CS-60C might be conjugated with monosaccharide(s), which

speculation would account for the failure to detect carbohydrate in CS-60C by the orcinol or Molisch reactions. Conjugated disaccharides or polysaccharides would be detectable with these reagents. And, indeed, polysaccharide from 0.9 to 35% was determined by the orcinol method in the series of allergens obtained in the electrophoretic fractionation of CS-1A (Spies *et al.*, 1941), all of which had the same antigenic specificity as CS-60C. Further evidence is required to determine the correctness of the Berrens and Bleumink (1965) contention on the hypothesized carbohydrate content of CS-60C and on its significance.

CURRENT RESEARCH ON MILK ALLERGENS AT DAIRY PRODUCTS LABORATORY, USDA

Lowell (1950), an allergist at Massachusetts General Hospital, stated: "There is perhaps no field of medicine in which more divergent views are held than in that of allergy to foods." Although much research on food allergy has been done since then, it seems that this opinion is still valid especially for milk, which is so widely used in infant feeding. The subject of milk allergy has recently been reviewed (Spies, 1973) and the reader is referred to this article for references and discussion which includes incidence, diagnosis, symptoms, breast *vs.* cow's milk in infant feeding, milk allergens and antibodies, crib deaths, prognosis, and current work at Dairy Products Laboratory (DPL), USDA.

The broad objective of the allergens investigations of DPL is control or inactivation of the allergens of milk so that this nutritious food will be acceptable to those who cannot tolerate it because of allergic response to ingestion of milk. The relatively low incidence of milk allergy in the total population (<0.1 to 7%) might seem too insignificant to justify research on milk allergy by DPL. However, the significance of this work is broader than these figures indicate at first glance. First, it is pediatric practice in many cases in families with a history of any allergy to eliminate milk from the diet of infants and children under 2 years of age, whether they are allergic or not, to minimize their chances of acquiring milk allergy. Second, some believe that prevention of milk allergy in infants tends to lessen their chances of acquiring other allergies in later childhood, after milk allergy, should they have acquired it, had disappeared. And third, milk contains well-characterized proteins which are ideally suited as model substances for studying all food allergies.

The present state of our knowledge requires, primarily, fundamental scientific investigations in an attempt to elucidate the mechanism of the allergic response to ingestion of milk before we can elaborate feasible procedures for the control of milk allergens. For the past 6 years we have been studying the immunological significance of pepsin hydrolytic products of milk proteins. The immunologic significance of enzyme hydrolytic products of ingested proteins has long been the subject of speculation and sporadic investigations. The consensus of the clinical significance of digestive products has been that they may be the cause of delayed clinical reactions of from 1 to 36 hr, or even days in some cases, after ingestion. Our work suggests that digestive products of milk may be the cause of immediate-type allergic response, possibly in addition to delayed responses.

We first demonstrated (Spies *et al.*, 1970) generation of a new antigen in the dialysate of the 8-min pepsin hydrolysate of each of four major milk proteins, namely β-lactoglobulin, α-lactalbumin, casein, and bovine serum albumin (BSA). The endo fraction of BSA contained a second new antigen. In our studies the term "new antigen" is defined as "an antigen with a specificity distinct from that of the protein from which it was generated."

The objective of later studies (Spies *et al.*, 1972a,b) was to determine whether one or several new antigens are generated by a simulated stomach digestion of β-lactoglobulin. In these studies β-lactoglobulin was hydrolyzed at six successive 8-min periods, during which approximately 90% of the protein was split into fragments with a molecular weight of 12,000 or less. Six dialysates (D1–D6) and six endo fractions (E1–E6) were separated and analyzed for the presence of new antigen using the Schultz–Dale technique and gel diffusion analysis, respectively. All of the dialysates contained common nonprecipitating new antigens. The first dialysate (D1) did not contain all of the new antigens common to the other five, (D2–D6), indicating at least two new antigens in the dialysates. Six precipitating new antigens were demonstrated in the endo fractions. By analogy to β-lactoglobulin, if pepsin hydrolysis generated at least eight new antigens from each of the known 12–14 antigenic proteins in milk (Hanson, 1959; Hanson and Johansson, 1959), the body immune system would be exposed to about 100 new antigens, all of which are potential allergens, on ingestion of milk. These results may explain why milk and other foods, in many cases, do not give skin reactions on persons who give an immediate allergic response on ingestion of the food. Such persons may be sensitive to these new antigens formed by pepsin in the stomach during digestion.

Although the sensitizing properties of these new antigens are unknown as yet, it seems likely that some of them, at least, might act as allergic sensitizers for food digestion products in a manner similar to that of other low-molecular weight substances. It was demonstrated (Spies *et al.*, 1970) that new antigen could be detected after only 1-, 2-, and 4-min pepsin hydrolysis of total milk protein. Later (Spies *et al.*, 1972a) it was apparent that common new antigens continue to be generated from β-lactoglobulin over a period of 48 min. Since absorption of immunologically significant amounts of allergens is known to occur in a few minutes, (Harten *et al.*, 1939; Spies *et al.*, 1945; Walzer, 1942; Walzer and Walzer, 1935), this continuous production of new antigens in the dialysates would tend to enhance their sensitizing potential.

In current studies three new antigens have been isolated from dialysate fractions of the pepsin hydrolysates of β-lactoglobulin, and their chemical composition and chemical and immunochemical properties were determined. Studies are underway to evaluate the fractions clinically to determine their allergenic significance, if any.

Demonstration of this multiplicity of new antigens (potential allergens) generated by pepsin hydrolysis of milk proteins as a simulated first step in digestion opens up a new area of study which should clarify an important aspect of the many perplexing aspects of food allergy in general and milk allergy in particular.

Two samples of lactose have been shown to contain traces of four antigens in the retentate from dialysis of large quantities of lactose which were not identifiable with known milk proteins (Spies, 1971). It was suggested that the increased skin reactivity attributed to the browning reaction product of β-lactoglobulin and lactose (Bleumink and Young, 1968) might be due to these antigens in lactose. This point requires clarification.

I have written this brief review of a complex subject from the vantage point of 37 years' research on the chemistry and other aspects of allergens. Justification for what may seem excessive reference to our work is twofold: first, the authority of first-hand knowledge; and second, feeling that possibly our most important contribution to allergen research was original demonstration and elucidation of the polysaccharidic protein nature of allergens in some natural systems, together with the concept of common antigenic and allergenic determinants, for which we wished to document and emphasize its importance in allergen isolation. It is especially important in the search for the elusive homogeneous allergen to realize that in some systems at least a common antigenic and allergenic specificity

may reside in a multitude of compounds rather than in one definite compound. Allergists are indebted to Dr. Berrens for his monograph on the chemistry of allergens with its detailed description of published works, his attempted physicochemical classification of allergens, and his proposed lysine–sugar conjugate as the universal allergenic determinant. Berrens' critical conclusions regarding the work of others are stimulating, but they should be verified by reference to the original works. Substantiation of the significance of the lysine–sugar universal allergenic determinant remains to be done. This linkage may have some significance but its universality is questionable. The clinical significance of the new antigens derived from milk proteins by pepsin digestion in relation to the mechanism of food allergy remains to be determined. Solution of problems of standardization of allergens and the continued development of *in vitro* or at least convenient nonhuman tests for allergens will contribute much to the advancement of knowledge of the chemistry of allergens.

Supplementary Material Available. Specific examples of factual errors and questionable interpretations found in the discussion on the glycoprotein nature of allergens conducted by the USDA group, along with corrections and replies, will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JAFC-73-000.

LITERATURE CITED

Aas, K., Elsayed, S. M., *J. Allergy* **44**, 333 (1969).
Augustin, R., *Immunology* **2**, 230 (1959).
Augustin, R., Hayward, B. J., *Immunology* **5**, 424 (1962).
Austen, K. F., in "Immunological Diseases," Samter, M., Ed., Little, Brown and Co., Boston, Mass., 1965, pp 211–225.
Barker, S. A., Cruickshank, C. N. D., Morris, J. H., *Biochem. Biophys. Acta* **74**, 239 (1967).
Bernton, H. S., Spies, J. R., Stevens, H., *J. Allergy* **13**, 289 (1942).
Berrens, L., *Allerg. Asthma* **8**, 75 (1962).
Berrens, L., *Recl. Trav. Chim. Pays-Bas* **83**, 429 (1964).
Berrens, L., Universiteitskliniek Voor Huidziekten, Utrecht, Holland, private communication, 1965.
Berrens, L., *Int. Arch. Allergy Appl. Immunol.* **29**, 575 (1966a).
Berrens, L., *Recl. Trav. Chim. Pays-Bas* **85**, 1117 (1966b).
Berrens, L., *Immunochemistry* **4**, 81 (1967).
Berrens, L., *Int. Arch. Allergy Appl. Immunol.* **34**, 81 (1968a).
Berrens, L., *Immunochemistry* **5**, 585 (1968b).
Berrens, L., in "Progress in Allergy," Vol. 14, Kallos, P., Waksman, B. H., Ed., Karger, Basel, 1970, pp 259–339.
Berrens, L., in "Monographs in Allergy," Vol. 7, Kallos, P., Hasek, M., Inderbitzen, T. M., Miescher, P. A., Waksman, B. H., Ed., Karger, Basel, 1971, pp 1–298.
Berrens, L., Bleumink, E., *Int. Arch. Allergy Appl. Immunol.* **28**, 150 (1965).
Berrens, L., Bleumink, E., *Recl. Trav. Chim. Pays-Bas.* **85**, 59 (1966).
Berrens, L., Morris, J. H., Young, E., *Dermatologica* **132**, 433 (1966).
Berrens, L., Versie, R., *Acta Allergol.* **22**, 347 (1967).
Berrens, L., Young, E., *Int. Arch. Allergy Appl. Immunol.* **22**, 51 (1963).
Bleumink, E., in "World Review of Nutrition and Dietetics," Vol. 12, Bourne, G. H., Ed., Karger, Basel, 1970, pp 505–570.
Bleumink, E., Berrens, L., Young, E., *Int. Arch. Allergy Appl. Immunol.* **31**, 25 (1967).
Bleumink, E., Young, E., *Int. Arch. Allergy Appl. Immunol.* **34**, 521 (1968).
Bleumink, E., Young, E., *Int. Arch. Allergy Appl. Immunol.* **35**, 1 (1969).
Coulson, E. J., Spies, J. R., *J. Immunol.* **46**, 367 (1943a).
Coulson, E. J., Spies, J. R., *J. Immunol.* **46**, 377 (1943b).
Coulson, E. J., Stevens, H., *J. Allergy* **11**, 537 (1940).
Coulson, E. J., Spies, J. R., Stevens, H., *J. Immunol.* **41**, 375 (1941).
Coulson, E. J., Spies, J. R., Stevens, H., *J. Immunol.* **46**, 347 (1943a).
Coulson, E. J., Spies, J. R., Stevens, H., *J. Immunol.* **47**, 443 (1943b).
Coulson, E. J., Spies, J. R., Stevens, H., *J. Immunol.* **49**, 99 (1944).
Coulson, E. J., Spies, J. R., Stevens, J., *J. Immunol.* **62**, 171 (1949).
Coulson, E. J., Spies, J. R., Stevens, H., Shimp, J. H., *J. Allergy* **21**, 34 (1950).
Gelfand, H. H., *J. Allergy* **14**, 203 (1943).
Goldfarb, A. R., *J. Immunol.* **100**, 902 (1968).
Goldstein, G. B., Heiner, D. C., *J. Allergy* **46**, 270 (1970).
Greenstein, J. P., Winitz, M., "Chemistry of the Amino Acids," Vol. 2, Wiley, New York, N. Y., 1961, p 1688.
Hanson, L. A., *Experientia* **15**, 471 (1959).
Hanson, L. A., Johansson, B., *Experientia* **15**, 377 (1959).
Hanson, L. A., Johansson, B. S., in "Milk Proteins. Chemistry and Molecular Biology," Vol. I, McKenzie, H. A., Ed., Academic Press, New York, N. Y., 1970, pp 45–123.
Harten, M., Gray, S., Livingston, S., Walzer, M., *J. Allergy* **10**, 478 (1939).
Herbertson, S., Porath, J., Colldahl, H., *Acta Chem. Scand.* **12**, 737 (1958).
Ishizaka, K., Ishizaka, T., *Ann. Allergy* **28**, 189 (1970).
Johnson, P., Marsh, D. G., *Eur. Polym. J.* **1**, 63 (1965).
Johnson, P., Marsh, D. G., *Immunochemistry* **3**, 101 (1966a).
Johnson, P., Marsh, D. G., *Nature (London)* **206**, 935 (1966b).
King, T. P., Norman, P. S., Lichtenstein, L. M., *Ann. Allergy* **25**, 541 (1967).
Levine, B. B., Redmond, A. P., *Int. Arch. Allergy Appl. Immunol.* **35**, 445 (1969).
Lowell, F. C., *J. Allergy* **21**, 563 (1950).
Morris, R. S., Spies, J. R., Coulson, E. J., *Arch. Biochem. Biophys.* **110**, 300 (1965).
Panzani, R., Layton, L. L., *Int. Arch. Allergy Appl. Immunol.* **22**, 350 (1963).
Prince, H. E., Halpin, L. J., Talbot, G., Etter, R. L., Raymer, W. J., Jackson, R. H., Bartlett, L. L., Mansmann, J. A., Frayser, L., Eisenberg, B. C., Morrow, M. B., Meyer, G. H., *Ann. Allergy* **29**, 259 (1961).
Raffel, S., in "Immunological Diseases," Samter, M., Alexander, H. L., Ed., Little, Brown and Co., Boston, Mass., 1965, pp 146–160.
Richter, M., Sehon, A. H., *J. Allergy* **31**, 111 (1960).
Robbins, K. C., Wu, H., Hsieh, B., *Immunochemistry* **3**, 71 (1966).
Shulman, S., in "Progress in Allergy," Vol. 12, Kallos, P., Waksman, B. H., Ed., Karger, Basel, 1968, pp 246–317.
Shulman, S., Bronson, P., Arbesman, C. E., *J. Allergy* **34**, 1 (1963).
Spies, J. R., *Ann. Allergy* **25**, 29 (1967).
Spies, J. R., *Proc. Soc. Exp. Biol. Med.* **137**, 211 (1971).
Spies, J. R., *J. Milk Food Technol.* **36**, 225 (1973).
Spies, J. R., Barron, J. K., *Ann. Allergy* **24**, 499 (1966).
Spies, J. R., Bernton, H. S., *J. Allergy* **33**, 73 (1962).
Spies, J. R., Bernton, H. S., Chambers, D. C., *J. Allergy* **31**, 162 (1960).
Spies, J. R., Bernton, H. S., Stevens, H., *J. Allergy* **10**, 113 (1939).
Spies, J. R., Bernton, H. S., Stevens, H., *J. Amer. Chem. Soc.* **62**, 2793 (1940a).
Spies, J. R., Bernton, H. S., Stevens, H., *J. Amer. Chem. Soc.* **63**, 2163 (1941).
Spies, J. R., Chambers, D. C., Bernton, H. S., Stevens, H., *J. Allergy* **14**, 7 (1942).
Spies, J. R., Chambers, D. C., Bernton, H. S., Stevens, H., *J. Allergy* **16**, 267 (1945).
Spies, J. R., Chambers, D. C., Coulson, E. J., *Arch. Biochem. Biophys.* **84**, 286 (1959).
Spies, J. R., Chambers, D. C., Coulson, E. J., Bernton, H. S., Stevens, H., *J. Allergy* **24**, 483 (1953).
Spies, J. R., Coulson, E. J., *J. Amer. Chem. Soc.* **65**, 1720 (1943).
Spies, J. R., Coulson, E. J., *J. Biol. Chem.* **239**, 1818 (1964).
Spies, J. R., Coulson, E. J., Bernton, H. S., Stevens, H., *J. Amer. Chem. Soc.* **62**, 1420 (1940b).
Spies, J. R., Coulson, E. J., Bernton, H. S., Wells, P. A., Stevens, H., *J. Agr. Food Chem.* **10**, 140 (1962).
Spies, J. R., Coulson, E. J., Chambers, D. C., Bernton, H. S., Stevens, H., *J. Amer. Chem. Soc.* **66**, 748 (1944a).
Spies, J. R., Coulson, E. J., Chambers, D. C., Bernton, H. S., Stevens, H., *J. Amer. Chem. Soc.* **66**, 2137 (1944b).
Spies, J. R., Coulson, E. J., Chambers, D. C., Bernton, H. S., Stevens, H., Shimp, J. H., *J. Amer. Chem. Soc.* **73**, 3995 (1951).
Spies, J. R., Stevan, M. A., Stein, W. J., *J. Allergy* **50**, 82 (1972a).
Spies, J. R., Stevan, M. A., Stein, W. J., *J. Allergy Clin. Immunol.* **50**, 82 (1972a).
Spies, J. R., Stevan, M. A., Stein, W. J., Coulson, E. J., *J. Allergy* **45**, 208 (1970).

Spies, J. R., Umberger, E. J., *J. Amer. Chem. Soc.* **64**, 1889 (1942).
Stanworth, D. R., *Biochem. J.* **65**, 582 (1957).
Stanworth, D. R., in "Advances in Immunology," Vol. 3, Dixon, F. J., Humphrey, J. H., Ed., Academic Press, New York, N. Y., 1963, pp 181–260.
Strejan, G., Campbell, D. H., *J. Immunol.* **101**, 628 (1968).
Underdown, B. J., Goodfriend, L., *Biochemistry* **8**, 980 (1969).
von Pirquet, C., *Muenchen. Med. Woechenschr.* **53**, 1457 (1906).
Walzer, M., *J. Allergy* **13**, 554 (1942).
Walzer, A., Walzer, M., *J. Allergy* **6**, 532 (1935).
Zucker, A., *Ann. Allergy* **23**, 335 (1965).

Received for review April 9, 1973. Accepted August 1, 1973. Presented in part at the Division of Agricultural and Food Chemistry Symposium on Toxic Proteins, Peptides, and Related Substances, 165th National Meeting of the American Chemical Society, Dallas, Tex., April 1973.